MAGE-1

Isolated TUMOR REJECTION ANTIGEN PRECURSORS, ~~TUMOR REJECTION ANTIGENS AND USES THEREOF~~

This application is a continuation-in-part of Serial Number 07/807,043, filed December 12, 1991, now U.S. Patent No. 5,342,774 which is a continuation-in-part of Serial Number 07/764,365, ~~764,364~~, filed September 23, 1991, now abandoned which is a continuation-in-part of Serial Number 07/728,838, filed July 9, 1991, now abandoned which is a continuation-in-part of Serial Number 07/705,702, filed May 23, 1991, and now abandoned.

FIELD OF THE INVENTION

This invention relates in general to the field of immunogenetics as applied to the study of oncology. More specifically, it relates to the study and analysis of mechanisms by which tumors are recognized by the organism's immune system such as through the presentation of so-called tumor rejection antigens, and the expression of what will be referred to herein as "tumor rejection antigen precursors".

BACKGROUND AND PRIOR ART

The study of the recognition or lack of recognition of cancer cells by a host organism has proceeded in many different directions. Understanding of the field presumes some understanding of both basic immunology and oncology.

Early research on mouse tumors revealed that these displayed molecules which led to rejection of tumor cells when transplanted into syngeneic animals. These molecules are "recognized" by T-cells in the recipient animal, and provoke a cytolytic T-cell response with lysis of the transplanted cells. This evidence was first obtained with tumors induced in vitro by chemical carcinogens, such as methylcholanthrene. The antigens expressed by the tumors and which elicited the T-cell response were found to be different for each tumor. See Prehn, et al., J. Natl. Canc. Inst. 18: 769-778 (1957); Klein et al., Cancer Res. 20: 1561-1572 (1960); Gross, Cancer Res. 3: 326-333 (1943), Basombrio, Cancer Res. 30: 2458-2462 (1970) for general teachings on inducing tumors with chemical carcinogens and differences in cell surface antigens. This class of antigens has come to be known as "tumor specific transplantation antigens" or "TSTAs". Following the observation of the presentation of such antigens when induced by chemical carcinogens, similar results were obtained when tumors were induced in vitro via ultraviolet radiation. See Kripke, J. Natl. Canc. Inst. 53: 333-1336 (1974).

While T-cell mediated immune responses were observed for the types of tumor described supra, spontaneous tumors were thought to be generally non-immunogenic. These were therefore believed not to present antigens which provoked a response to the tumor in the tumor carrying subject. See Hewitt, et al., Brit. J. Cancer 33: 241-259 (1976).

The family of tum^- antigen presenting cell lines are immunogenic variants obtained by mutagenesis of mouse tumor cells or cell lines, as described by Boon et al., J. Exp. Med. 152: 1184-1193 (1980), the disclosure of which is incorporated by reference. To elaborate, tum^- antigens are obtained by mutating tumor cells which do not generate an immune response in syngeneic mice and will form tumors (i.e., "tum^+" cells). When these tum^+ cells are mutagenized, they are rejected by syngeneic mice, and fail to form tumors (thus "tum^-"). See Boon et al., Proc. Natl. Acad. Sci. USA 74: 272 (1977), the disclosure of which is incorporated by reference. Many tumor types have been shown to exhibit this phenomenon. See, e.g., Frost et al., Cancer Res. 43: 125 (1983).

It appears that tum^- variants fail to form progressive tumors because they elicit an immune rejection process. The evidence in favor of this hypothesis includes the ability of "tum^-" variants of tumors, i.e., those which do not normally form tumors, to do so in mice with immune systems suppressed by sublethal irradiation, Van Pel et al., Proc. Natl, Acad. Sci. USA 76: 5282-5285 (1979); and the observation that intraperitoneally injected tum^- cells of mastocytoma P815 multiply exponentially for 12-15 days, and then are eliminated in only a few days in the midst of an influx of lymphocytes and macrophages (Uyttenhove et al., J. Exp. Med. 152: 1175-1183 (1980)). Further evidence includes the observation that mice acquire an immune memory

which permits them to resist subsequent challenge to the same tum- variant, even when immunosuppressiv amounts of radiation are administered with the following challenge of cells (Boon et al., Proc. Natl, Acad. Sci. USA 74: 272-275 (1977); Van Pel et al., supra; Uyttenhove et al., supra).

Later research found that when spontaneous tumors were subjected to mutagenesis, immunogenic variants were produced which did generate a response. Indeed, these variants were able to elicit an immune protective response against the original tumor. See Van Pel et al., J. Exp. Med. 157: 1992-2001 (1983). Thus, it has been shown that it is possible to elicit presentation of a so-called "tumor rejection antigen" in a tumor which is a target for a syngeneic rejection response. Similar results have been obtained when foreign genes have been transfected into spontaneous tumors. See ~~Fearson~~ Fearon et al., Cancer Res. 48: 2975-1980 (1988) in this regard.

A class of antigens has been recognized which are presented on the surface of tumor cells and are recognized by cytotoxic T cells, leading to lysis. This class of antigens will be referred to as "tumor rejection antigens" or "TRAs" hereafter. TRAs may or may not elicit antibody responses. The extent to which these antigens have been studied, has been via cytolytic T cell characterization studies, in vitro i.e., the study of the identification of the antigen by a particular cytolytic T cell ("CTL" hereafter) subset. The subset proliferates upon recognition of the presented tumor rejection antigen, and

the cells presenting the antigen ar lysed. Characterization studies have identified CTL clones which specifically lyse cells expressing the antigens. Examples of this work may be found in Levy et al., Adv. Cancer Res. 24: 1-59 (1977); Boon et al., J. Exp. Med. 152: 1184-1193 (1980); Brunner et al., J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 124: 1627-1634 (1980); Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982); Palladino et al., Canc. Res. 47: 5074-5079 (1987). This type of analysis is required for other types of antigens recognized by CTLs, including minor histocompatibility antigens, the male specific H-Y antigens, and a class of antigens, referred to as "tum-" antigens, and discussed herein.

A tumor exemplary of the subject matter described supra is known as P815. See DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988); Szikora et al., EMBO J 9: 1041-1050 (1990), and Sibille et al., J. Exp. Med. 172: 35-45 (1990), the disclosures of which are incorporated by reference. The P815 tumor is a mastocytoma, induced in a DBA/2 mouse with methylcholanthrene and cultured as both an in vitro tumor and a cell line. The P815 line has generated many tum^- variants following mutagenesis, including variants referred to as P91A (DePlaen, supra), 35B (Szikora, supra), and P198 (Sibille, supra). In contrast to tumor rejection antigens - and this is a key distinction - the tum^- antigens are

only present after the tumor cells are mutagenized. Tumor rejection antigens are present on cells of a given tumor without mutagenesis. Hence, with reference to th literature, a cell line can be tum^+, such as the line referred to as "P1", and can be provoked to produce tum^- variants. Since the tum^- phenotype differs from that of the parent cell line, one expects a difference in the DNA of tum^- cell lines as compared to their tum^+ parental lines, and this difference can be exploited to locate the gene of interest in tum^- cells. As a result, it was found that genes of tum^- variants such as P91A, 35B and P198 differ from their normal alleles by point mutations in the coding regions of the gene. See Szikora and Sibille, supra, and Lurquin et al., Cell 58: 293-303 (1989). This has proved not to be the case with the TRAs of this invention. These papers also demonstrated that peptides derived from the tum^- antigen are presented by the L^d molecule for recognition by CTLs. P91A is presented by L^d, P35 by D^d and P198 by K^d.

It has now been found that the genes which code for the molecules which are processed to form the presentation tumor rejection antigens (referred to as "tumor rejection antigen precursors", "precursor molecules" or "TRAPs" hereafter), are not expressed in most normal adult tissues but are expressed in tumor cells. Genes which code for the TRAPs have now been isolated and cloned, and represent a portion of the invention disclosed herein.

The gene is useful as a source for the isolated and purified tumor rejection antigen precursor and the TRA themselves, either of which can b used as an agent for treating the cancer for which the antigen is a "marker", as well as in various diagnostic and surveillance approaches to oncology, discussed infra. It is known, for example, that tum$^-$ cells can be used to generate CTLs which lyse cells presenting different tum$^-$ antigens as well as tum$^+$ cells. See, e.g., Maryanski et al., Eur. J. Immunol 12: 401 (1982); and Van den Eynde et al., Modern Trends in Leukemia IX (June 1990), the disclosures of which are incorporated by reference. The tumor rejection antigen precursor may be expressed in cells transfected by the gene, and then used to generate an immune response against a tumor of interest.

In the parallel case of human neoplasms, it has been observed that autologous mixed lymphocyte-tumor cell cultures ("MLTC" hereafter) frequently generate responder lymphocytes which lyse autologous tumor cells and do not lyse natural killer targets, autologous EBV-transformed B cells, or autologous fibroblasts (see Anichini et al., Immunol. Today 8: 385-389 (1987)). This response has been particularly well studied for melanomas, and MLTC have been carried out either with peripheral blood cells or with tumor infiltrating lymphocytes. Examples of the literature in this area including Knuth et al., Proc. Natl. Acad. Sci. USA 86: 2804-2802 (1984); Mukherji et al., J. Exp. Med.

158: 240 (1983); Hérin et all, Int. J. Canc. 39: 390-396 (1987); Topalian et al, J. Clin. Oncol 6: 839-853 (1988). Stable cytotoxic T cell clones ("CTLs" hereafter) have been derived from MLTC responder cells, and these clones are specific for the tumor cells. See Mukherji et al., supra, Hérin et all, supra, Knuth et al., supra. The antigens recognized on tumor cells by these autologous CTLs do not appear to represent a cultural artifact, since they are found on fresh tumor cells. Topalian et al., supra; Degiovanni et al., Eur. J. Immunol. 20: 1865-1868 (1990). These observations, coupled with the techniques used herein to isolate the genes for specific murine tumor rejection antigen precursors, have led to the isolation of nucleic acid sequences coding for tumor rejection antigen precursors of TRAs presented on human tumors. It is now possible to isolate the nucleic acid sequences which code for tumor rejection antigen precursors, including, but not being limited to those most characteristic of a particular tumor, with ramifications that are described infra. These isolated nucleic acid sequences for human tumor rejection antigen precursors and applications thereof, as described infra, are also the subject of this invention.

These and various other aspects of the invention are elaborated upon in the disclosure which follows.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts detection of transfectants expressing antigen P815A.

Figure 2 shows the sensitivity of clones P1.HTR, PO.HTR, genomic transfectant P1A.T2 and cosmid transfectant P1A.TC3.1 to lysis by various CTLs, as determined by chromium release assays.

Figure 3 is a restriction map of cosmid C1A.3.1.

Figure 4 shows Northern Blot analysis of expression of gene P1A.

Figure 5 sets forth the structure of gene P1A with its restriction sites.

Figure 6 shows the results obtained when cells were transfected with the gene from P1A, either isolated from P815 or normal cells and then tested with CTL lysis.

Figure 7 shows lytic studies using mast cell line L138. 8A.

Figure 8 is a map of subfragments of the 2.4 kb antigen E fragment sequence which also express the antigen.

Figure 9 shows homology of sections of exon 3 from genes mage 1, 2 and 3.

Figure 10 shows the result of Northern blots for MAGE genes on various tissues.

Figur 11H and 11B presents the data of Figure 13 in table form.

Figure 12 shows Southern Blot experiments using the various human melanoma cell lines employed in this application.

Figure 13 is a generalized schematic of the expression of MAGE 1, 2 and 3 genes by tumor and normal tissues.

BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO: 1 is cDNA for part of gene P1A.

SEQ ID NO: 2 presents coding region of cDNA for gene P1A.

SEQ ID NO: 3 shows non coding DNA for P1A cDNA which is 3' to the coding region of SEQ ID NO: 2.

SEQ ID NO: 4 is the entire sequence of cDNA for P1A.

SEQ ID NO: 5 is the genomic DNA sequence for P1A.

SEQ ID NO: 6 shows the amino acid sequence for the antigenic peptides for P1A TRA. The sequence is for cells which are A^+ B^+, i.e., express both the A and B antigens.

SEQ ID NO: 7 is a nucleic acid sequence coding for antigen E.

SEQ ID NO: 8 is a nucleic acid sequence coding for MAGE-1.

SEQ ID NO: 9 is the gene for MAGE-2.

SEQ ID NO: 10 is the gene for MAGE-21.

SEQ ID NO: 11 is cDNA for MAGE-3.

SEQ ID NO: 12 is the gene for MAGE-31.

SEQ ID NO: 13 is the gene for MAGE-4.

SEQ ID NO: 14 is the gene for MAGE-41.

SEQ ID NO: 15 is cDNA for MAGE-4.

SEQ ID NO: 16 is cDNA for MAGE-5.

SEQ ID NO: 17 is genomic DNA for MAGE-51.

SEQ ID NO: 18 is cDNA for MAGE-6.

SEQ ID NO: 19 is genomic DNA for MAGE-7.

SEQ ID NO: 20 is genomic DNA for MAGE-8.

SEQ ID NO: 21 is genomic DNA for MAGE-9.

SEQ ID NO: 22 is genomic DNA for MAGE-10.

SEQ ID NO: 23 is genomic DNA for MAGE-11.

SEQ ID NO: 24 is genomic DNA for smage-I.

SEQ ID NO: 25 is genomic DNA for smage-II.

Ins H'

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

Many different "MAGE" genes have been identified, as will be s n from the sequences which follow the application. The protocols described in the following

examples were used to isolat these genes and cDNA sequences.

"MAGE" as used her in refers to a nucleic acid sequence isolated from human cells. The acronym "smage" is used to describe sequences of murine origin.

When "TRAP" or "TRAs" are discussed herein as being specific to a tumor type, this means that the molecule under consideration is associated with that type of tumor, although not necessarily to the exclusion of other tumor types.

Example 1

In order to identify and isolate the gene coding for antigen P815A, gene transfection was used. This approach requires both a source of the gene, and a recipient cell line. Highly transfectable cell line P1.HTR was the starting material for the recipient, but it could not be used without further treatment, as it presents "antigen A", one of four recognized P815 tumor antigens. See Van Pel et al., Molecular Genetics 11: 467-475 (1985). Thus, screening experiments were carried out to isolate cell lines which did not express the antigen and which nonetheless possessed P1.HTR's desirable qualities.

To do this, P1.HTR was screened with CTLs which were specific for each of tumor antigens A, B, C and D. Such CTLs are described by Uyttenhove et al., J. Exp. Med. 157: 1040-1052 (1983).

To carry out the selection, 10^6 cells of P1.HTR were mixed with 2-4x10^6 cells of the CTL clone in a round bottom tube in 2 ml of medium, and centrifuged for three minutes at 150xg. After four hours at 37°C, the cells were washed and resuspended in 10 ml of medium, following Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982). Additional information on the CTL assay and screening protocol, in general may be found in Boon et al., J. Exp. Med. 152: 1184-1193 (1980), and Maryanski et al., Eur. J. Immunol. 12: 406-412 (1982), the disclosure of which are incorporated by reference.

When these selections were carried out, a cell line variant was found which expressed neither antigen A or B. Additional selections with CTLs specific for antigen C then yielded a variant which also lacked antigen C. Please see figure 2 for a summary of the results of these screenings. The variant PO.HTR is negative for antigens A, B and C, and was therefore chosen for the transfection experiments.

The cell line PO.HTR has been deposited in accordance with the Budapest Treaty at the Institute Pasteur Collection Nationale De Cultures De Microorganismes, 28, Rue de Docteur Roux, 75724 Paris France, and has accession number I-1117.

This methodology is adaptable to secure other cell lines which are variants of a cell type which normally presents at least one of the four recognized P815 tumor antigens, i.e., antigens A, B, C and D, where the variants

present none of antigens A, B and C. P1.HTR is a mastocytoma cell line, so it will be seen that the protocol enables the isolation of biologically pure mastocytoma cell lines which express none of P815 antigens A, B and C, but which are highly transfectable. Other tumor types may also be screened in this fashion to secure desired, biologically pure cell lines. The resulting cell lines should be at least as transfectable with foreign DNA as is P1.HTR, and should be selected so as to not express a specific antigen.

Example 2

Previous work reported by DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988) the disclosure of which is incorporated by reference herein had shown the efficacy of using cosmid library transfection to recover genes coding for tum^- antigens.

Selective plasmid and genomic DNA of P1.HTR were prepared, following Wölfel et al., Immunogenetics 26: 178-187 (1987). The transfection procedure followed Corsaro et al., Somatic Cell Molec. Genet 7: 603-616 (1981), with some modification. Briefly, 60 μg of cellular DNA and 3 μg of DNA of plasmid pHMR272, described by Bernard et al., Exp. Cell. Biol. 158: 237-243 (1985) were mixed. This plasmid confers hygromycin resistance upon recipient cells, and therefore provides a convenient way to screen for transfectants. The mixed DNA was combined with 940 ul of 1 mM Tris-HCl (pH 7.5), 0.1 mM EDTA; and 310 ul 1M $CaCl_2$.

The solution was added slowly, and under constant agitation to 1.25 ml of 50 mM Hepes, 280 mM NaCl, 1.5 mM Na_2HPO_4, adjusted to pH 7.1 with NaOH. Calcium phosphate - DNA precipitates were allowed to form for 30-45 minutes at room temperature. Following this, fifteen groups of PO.HTR cells ($5x10^6$) per group were centrifuged for 10 minutes at 400 g. Supernatants were removed, and pellets were resuspended directly into the medium containing the DNA precipitates. This mixture was incubated for 20 minutes at 37°C, after which it was added to an 80 cm^2 tissue culture flask containing 22.5 ml DMEM, supplemented with 10% fetal calf serum. After 24 hours, medium was replaced. Forty-eight hours after transfection, cells were collected and counted. Transfected cells were selected in mass culture using culture medium supplemented with hygromycin B (350 ug/ml). This treatment selected cells for hygromycin resistance.

For each group, two flasks were prepared, each containing $8x10^6$ cells in 40 ml of medium. In order to estimate the number of transfectants, $1x10^6$ cells from each group were plated in 5 ml DMEM with 10% fetal calf serum (FCS), 0.4% bactoagar, and 300 ug/ml hygromycin B. The colonies were then counted 12 days later. Two independent determinations were carried out and the average taken. This was multiplied by 5 to estimate the number of transfectants in the corresponding group. Correction had

to be made for the cloning efficiency of P815 cells, known to be about 0.3.

Example 3

Eight days after transfection as described in example 2, _supra_, antibiotic resistant transfectants were separated from dead cells, using density centrifugation with Ficoll-Paque. These cells were maintained in non-selective medium for 1 or 2 days. The cells were plated in 96 well microplates (round bottom), at 30 cells/microwell in 200 ul of culture medium. Anywhere from 100-400 microwells were prepared, depending on the number of transfectants prepared. Agar colony tests gave estimates of 500-3000. After 5 days, the wells contained about $6x10^4$ cells and replicate plates were prepared by transferring 1/10 of the wells to microplates which were then incubated at 30°C. One day later, master plates were centrifuged, medium removed, and 750 CTLs against P815 antigen A (CTL-P1:5) were added to each well together with 10^6 irradiated syngeneic feeder spleen cells in CTL culture medium containing 40 U/ml recombinant human IL-2, and HAT medium to kill stimulator cells. Six days later, plates were examined visually to identify wells where CTLs had proliferated. Where plates showed proliferating microcultures, aliquots of 100 ul of the wells were transferred to another plate containing ^{51}Cr labeled P1.HTR target cells ($2x10^3$ - $4x10^3$ per well), and chromium release

was measured after 4 hours. Replicate microcultures corresponding to those showing high CTL activity were expanded and cloned by limited dilution in DMEM with 10% FCS. Five days later, about 200 clones were collected and screened with the CTL.P1:5 cell line, described supra, in a visual lysis assay. See figure 1A for these results.

In these experiments, three of the fifteen groups of transfectants yielded a few positive microcultures. These microcultures were tested for lytic activity against P1.HTR, as described supra. Most of the microcultures where proliferation was observed showed lytic activity. This activity was well above background, as shown in figure 1B. This figure summarizes data wherein two groups of cells (groups "5" and "14"), 400 and 300 microwells were seeded with 30 hygromycin resistant transfected cells. Amplification and duplication of the microcultures was followed by addition of anti-A CTL P1:5. Six days later, lytic activity against P1.HTR was tested. In the figure, each point represents lytic activity of a single microculture.

Duplicate microcultures corresponding to several positive wells were subcloned, and more than 1% of the subclones were found to be lysed by anti-A CTL. Thus, three independent transfectants expressing P815A were obtained from 33,000 hygromycin resistant transfectants. One of these lines, referred to hereafter as P1A.T2 was tested further.

The relevant antigen profile of P1A.T2 is shown in figure 2, this being obtained via anti-CTL assays of the type described supra.

Example 4

The CTL assays carried out for P1A.T2 demonstrated that it presented antigen A ("P815A"), and therefore had received the gene from P1.HTR. To that end, this cell line was used as a source for the gene for the antigen precursor in the following experiments.

Prior work had shown that genes coding for tum$^-$ antigens could be recovered directly from transfectants obtained with a cosmid library. See DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988). This procedure was followed for recovery of the P815 gene.

Total genomic DNA of P1A.T2 was partially digested with restriction endonuclease Sau 3A1, and fractionated by NaCl density gradient ultracentrifugation to enrich for 35-50 kb DNA fragments, following Grosveld et al., Gene 10: 6715-6732 (1982). These fragments were ligated to cosmid arms of C2RB, described by Bates et al., Gene 26: 137-146 (1983), the disclosure of which is incorporated by reference. These cosmid arms had been obtained by cleavage with SmaI and treatment with calf intestinal phosphatase, followed by digestion with BamHI. Ligated DNA was packaged into lambda phage components, and titrated on E. coli ED 8767, following Grosveld et al., supra. Approximately $9x10^5$

ampicillin resistant colonies were obtained per microgram of DNA insert.

The cosmid groups were amplified by mixing 30,000 independent cosmids with 2 ml of ED 8767 in 10 mM $MgCl_2$, incubated 20 minutes at 37°C, diluted with 20 ml of Luria Bertani ("LB") medium, followed by incubation for one hour. This suspension was titrated and used to inoculate 1 liter of LB medium in the presence of ampicillin (50 ug/ml). At a bacterial concentration of $2x10^8$ cells/ml (OD_{600}=0.8), a 10 ml aliquot was frozen, and 200 ug/ml chloramphenicol was added to the culture for overnight incubation. Total cosmid DNA was isolated by alkaline lysis procedure, and purified on CsCl gradient.

In these experiments, a library of 650,000 cosmids was prepared. The amplification protocol involved the use of 21 groups of approximately 30,000 cosmids.

Example 5

Using the twenty-one groups of cosmids alluded to supra, (60 ug) and 4 ug of pHMR272, described supra, groups of $5x10^6$ PO.HTR cells were used as transfectant hosts. Transfection was carried out in the same manner as described in the preceding experiments. An average of 3000 transfectants per group were tested for antigen presentation, again using CTL assays as described. One group of cosmids repeatedly yielded positive transfectants, at a frequency of about 1/5,000 drug resistant

transfectants. The transfectants, as with P1A.T2, also showed expression of both antigen A and B. The pattern of expression of transfectant P1A.TC3.1 is shown in figure 2.

Example 6

As indicated in Example 5, supra, three independent cosmid transfected cells presenting P815A antigen were isolated. The DNA of these transfectants was isolated and packaged directly with lambda phage extracts, following DePlaen et al., Proc. Natl. Acad. Sci. USA 85: 2274-2278 (1988). The resulting product was titrated on E. coli ED 8767 with ampicillin selection, as in Example 5. Similarly, amplification of the cosmids and transfection followed Example 5, again using PO.HTR.

High frequencies of transfection were observed, as described in Table 1, which follows:

Table 1. Transfer of the expression of antigen P815A by cosmids obtained by direct packaging

Transfectant obtained with the cosmid library	No. of cosmids obtained by direct packaging of 0.5 µg of DNA	No. of transfectants expressing P815A / no. of $H-2B^d$ transfectants
TC3.1	32	87/192
TC3.2	32000	49/384
TC3.3	44	25/72

The cosmids were analyzed with restriction enzymes and it was found that directly packaged transfectant P1A.TC3.1 contained 32 cosmids, 7 of which were different. Each of these 7 cosmids was transfected into PO.HTR, in the manner described <u>supra</u>, and again, following the protocols described above, transfectants were studied for presentation of P815A. Four of the cosmid transfectants showed P815A presentation and, as with all experiments described herein, P815B was co-expressed.

Of the four cosmids showing presentation of the two antigens, cosmid C1A.3.1 was only 16.7 kilobases long, and was selected for further analysis as described <u>infra</u>.

The cosmid C1A.3.1 was subjected to restriction endonuclease analysis, yielding the map shown in Figure 3.

All EcoRI fragments were transfected, again using the above described protocols, and only the 7.4 kilobase fragment produced a transfectant that anti-A CTLs could lyse. Similar experiments were carried out on the PstI fragments, and only a 4.1 kb fragment fully contained within the 7.4 kb EcoRI fragment produced lysable transfectants.

This fragment (i.e., the 4.1 kb PstI fragment), was digested with SmaI, giving a 2.3 kb fragment which also yielded host cells presenting antigens A and B after transfection. Finally, a fragment 900 bases long, secured with SmaI/XbaI, also transferred expression of the precursors of these two antigens, i.e., the transfected host cell presented both antigen A and antigen B.

Exampl 7

The 900 base fragment described above was used as a probe to detect the expression of the P815A gene in parent cell line P1.HTR. To accomplish this, total cellular RNA was first isolated using the guanidine-isothiocyanate procedure of Davis et al., Basic Methods In Molecular Biology (Elseview Science Publishing Co, New York) (1986). The same reference was the source of the method used to isolate and purify polyA$^+$ mRNA using oligodT cellulose column chromatography.

Samples were then subjected to Northern Blot analysis. RNA samples were fractionated on 1% agarose gels containing 0.66 M formaldehyde. The gels were treated with 10xSSC (SSC: 0.15 M NaCl; 0.015 M sodium citrate, pH 7.0) for 30 minutes before overnight blotting on nitrocellulose membranes. These were baked for two hours at 80°C, after which the membranes were prehybridized for 15 minutes at 60°C in a solution containing 10% dextran sulfate, 1% SDS and 1M NaCl. Hybridization was then carried out using denatured probe (the 900 base fragment), together with 100 ug/ml salmon sperm DNA.

When this protocol was carried out using P1.HTR poly A$^+$ RNA, a band of 1.2 kb and two fainter bands were identified, as shown in Figure 4, lane 1 (6 ug of the RNA).

The same probe was used to screen a cDNA library, prepared from poly-A$^+$ RNA from the cell line. This yielded

a clone with a 1kb insert, suggesting a missing 5′ end. The Northern blots for the cDNA are not shown.

Hybridization experiments in each case were carried out overnight at 60°C. The blots were washed twice at room temperature with 2xSSC and twice at 60°C with 2xSSC supplemented with 1% SDS.

The foregoing experiments delineated the DNA expressing the P815A antigen precursor sufficiently to allow sequencing, using the well known Sanger dideoxy chain termination method. This was carried out on clones generated using a variety of restriction endonucleases and by specific priming with synthetic oligonucleotide primers. The results for exons of the gene are set forth in sequence id no: 4.

Example 8

The Northern analysis described supra suggested that the 5′ end of the cDNA was missing. To obtain this sequence, cDNA was prepared from P1.HTR RNA using a primer corresponding to positions 320-303. The sequence was then amplified using the polymerase chain reaction using a 3′ primer corresponding to positions 286-266 and a 5′ primer described by Frohman et al., Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988). A band of the expected size (270 bases) was found, which hybridized to the 900 bp SmaI/XbaI fragment described supra on a Southern blot. Following cloning into m13tg 130 λ tg 131, the small, 270 bp fragment was sequenced. The sequence is shown in sequence id no: 1.

Exampl 9

Following the procurement of the sequences described in Examples 7 and 8 and depicted in seq id no: 4, a 5.7 kb region of cosmid C1A.3.1 was sequenced. This fragment was known to contain the 900 base fragment which expressed P815A in transfectants. This experiment permitted delineation of introns and exons, since the cosmid is genomic in origin.

The delineated structure of the gene is shown in figure 5. Together with seq id no: 4, these data show that the gene for the antigen precursor, referred to as "P1A" hereafter, is approximately 5 kilobases long and contains 3 exons. An ORF for a protein of 224 amino acids starts in exon 1, ending in exon 2. The 900 base pair fragment which transfers expression of precursors for antigens A and B only contains exon 1. The promoter region contains a CAAT box, as indicated in seq. id no: 1, and an enhancer sequence. This latter feature has been observed in promoters of most MHC class I genes, as observed by Geraghty et al., J. Exp. Med 171: 1-18 (1990); Kimura et al., Cell 44: 261-272 (1986).

A computer homology search was carried out, using program FASTA with K-triple parameters of 3 and 6, as suggested by Lipman et al., Science 227: 1435-1441 (1985), and using Genbank database release 65 (October 1990). No homology was found except for a stretch of 95 bases corresponding to part of an acid region coded by exon 1 (positions 524-618), which is similar to sequences coding

for acidic regions in mouse nucleolar protein NO38/B23, as described by Bourbon et al., Mol. Biol. 200: 627-638 (1988), and Schmidt-Zachmann et al., Chromosoma 96: 417-426 (1988). Fifty six of 95 bases were identical. In order to test whether these homologies were the reason for cross hybridizing, experiments were carried out using a mouse spleen cDNA library screened with the 900 base fragment. cDNA clones corresponding closely to the sizes of the cross hybridizing bands were obtained. These were partially sequenced, and the 2.6 kb cDNA was found to correspond exactly to reported cDNA sequence of mouse nucleolin, while the 1.5 kb cDNA corresponded to mouse nucleolar protein NO38/B23.

Analysis of the nucleotide sequence of the gene, referred to as "P1A" hereafter, suggests that its coded product has a molecular mass of 25 kd. Analysis of the sequence id no: 4 shows a potential nuclear targeting signal at residues 5-9 (Dingwall et al., Ann. Rev. Cell Biol. 2: 367-390 (1986)), as well as a large acidic domain at positions 83-118. As indicated supra, this contains the region of homology between P1A and the two nucleolar proteins. A putative phosphorylation site can be found at position 125 (serine). Also, a second acidic domain is found close to the C-terminus as an uninterrupted stretch of 14 glutamate residues. A similar C-terminal structure has been found by Kessel et al. Proc. Natl. Acad. Sci. USA 84: 5306-5310 (1987), in a murine homeodomain protein having nuclear localization.

In studies comparing the sequence of gene P1A to the sequences for P91A, 35B and P198, no similarities were found, showing that P1A is indicative of a different class of genes and antigens.

Example 10

With the P1A probe and sequence in hand, investigations were carried out to determine whether the gene present in normal tissue was identical to that expressed by the tumor. To do this, phage libraries were prepared, using lambda zapII 10 and genomic DNA of DBA2 murine kidney cells. P1A was used as a probe. Hybridization conditions were as described supra, and a hybridizing clone was found. The clone contained exons one and two of the P1A gene, and corresponded to positions -0.7 to 3.8 of figure 5. Following localization of this sequence, PCR amplification was carried out to obtain the sequence corresponding to 3.8 to 4.5 of figure 5.

Sequence analysis was carried out, and no differences were found between the gene from normal kidneys and the P1A gene as obtained from the P815 tumor cells.

In further experiments, the gene as found in DBA/2 kidney cells was transfected into PO.HTR, as described supra. These experiments, presented pictorially in figure 7, showed that antigens A and B were expressed as efficiently by the kidney gene isolated from normal kidney cells as with the P1A gene isolated from normal kidney cells.

These experiments lead to the conclusion that the gene coding for the tumor rejection antigen precursor is a gene that does not result from a mutation; rather, it would appear that the gene is the same as one present in normal cells, but is not expressed therein. The ramifications of this finding are important, and are discussed infra.

In studies not elaborated upon herein, it was found that variants of the gene were available. Some cells were "P1A^{-}B^{+}", rather than the normal "P1A". The only difference between these is a point mutation in exon 1, with the 18th triplet coding for Ala in the variant instead of Val.

Example 11

Additional experiments were carried out with other cell types. Following the protocols described for Northern blot hybridizations supra, RNA of normal liver and spleen cells was tested to determine if a transcript of the P1A gene could be found. The Northern blot data are presented in figure 4 and, as can be seen, there is no evidence of expression.

The murine P815 cell line from which P1A was isolated is a mastocytoma. Therefore, mast cell lines were studied to determine if they expressed the gene. Mast cell line MC/9, described by Nabel et al., Cell 23: 19-28 (1981), and short term cultures of bone marrow derived mast cells were tested in the manner described supra (Northern blotting), but no transcript was found. In contrast when a Balb/C derived IL-3 dependent cell line L138.8A (Hültner t al.,

J. Immunol. 142: 3440-3446 (1989)) was tested, a strong signal was found. The mast cell work is shown in figure 4.

It is known that both BALB/C and DBA/2 mice share H-2^d haplotype, and thus it was possible to test sensitivity to lysis using the CTLs described supra. Figure 8 shows these results, which essentially prove that anti-A and anti-B CTLs lysed the cells strongly, whereas anti-C and anti-D lines did not.

Further tests were carried out on other murine tumor cell lines, i.e., teratocarcinoma cell line PCC4 (Boon et al., Proc. Natl. Acad. Sci. USA 74: 272-275 (1977), and leukemias LEC and WEH1-3B. Expression could not be detected in any of these samples.

Example 12

The actual presentation of the P1A antigen by MHC molecules was of interest. To test this, cosmid C1A.3.1 was transfected into fibroblast cell line DAP, which shows phenotype H-2^k. The cell lines were transfected with genes expressing one of the K^d, D^d, and L^d antigen. Following transfection with both the cosmid and the MHC gene, lysis with CTLs was studied, again as described supra. These studies, summarized in Table 2, show that L^d is required for presentation of the P1A antigens A and B.

Table 2. H-2-restriction of antigens P815A and P815B

Recipient cell*	No of clones lysed by the CTL/ no. of H^+B^+ clones*	
	CTL anti-A	CTL anti-B
DAP ($H\text{-}2^k$)	0/208	0/194
DAP + K^d	0/165	0/162
DAP + D^d	0/157	0/129
DAP + L^d	25/33	15/20

*Cosmid C1A.3.1 containing the entire P1A gene was transfected in DAP cells previously transfected with $H\text{-}2^d$ class I genes as indicated.
*Independent drug-resistant colonies were tested for lysis by anti-A or anti-B CTL in a visual assay.

The observation that one may associate presentation of a tumor rejection antigen with a particular MHC molecule was confirmed in experiments with human cells and HLA molecules, as elaborated upon infra.

Example 13

Using the sequence of the P1A gene as well as the amino acid sequence derivable therefrom, antigenic peptides which were A^+ B^+ (i.e., characteristic of cells which express both the A and B antigens), and those which are A^- B^+ were identified. The peptide is presented in Figure 10. This peptide when administered to samples of PO.HTR cells

in the presence of CTL cell lines specific to cells presenting it, led to lysis of the PO.HTR cells, lending support to the view that peptides based on the product expressed by the gene can be used as vaccines.

Example 14

The human melanoma cell line referred to hereafter as MZ2-MEL is not a clonal cell line. It expresses four stable antigens recognized by autologous CTLs, known as antigens "D, E, F, and A". In addition, two other antigens "B" and "C" are expressed by some sublines of the tumor. CTL clones specific for these six antigens are described by Van den Eynde et al., Int. J. Canc. 44: 634-640 (1989). Among the recognized subclones of MZ2-MEL are MEL.43, MEL3.0 and MEL3.1. (Van den Eynde et al., supra). Cell line MEL3.1 expresses antigen E, as determined by CTL studies as described for P815 variants, supra, so it was chosen as a source for the nucleic acid sequence expressing the antigen precursor.

In isolating the pertinent nucleic acid sequence for a tumor rejection antigen precursor, the techniques developed supra, showed that a recipient cell is needed which fulfills two criteria: (i) the recipient cell must not express the TRAP of interest under normal conditions, and (ii) it must express the relevant class I HLA molecul . Also, the recipient cell must have a high transfection frequency, i.e., it must be a "good" recipient.

In order to secure such a cell line, the clonal sublin ME3.1 was subjected to repeated selection with anti-E CTL 82/30 as described by Van den Eynde, supra. The repeated cycles of selection led to isolation of subclone MZ2-MEL-2.2 isc E^-. This subclone is also $HPRT^-$, (i.e., sensitive to HAT medium: 10^{-4} M hypoxanthine, 3.8×10^{-7} aminopterine, 1.6×10^{-5} M 2-deoxythymidine). The subclone is referred to as "MEL-2.2" for simplicity hereafter.

Example 15

The genomic DNA of MEL3.0 was prepared following Wölfel et al., Immunogenetics 26: 178-187 (1987), the disclosure of which is incorporated by reference. The plasmid pSVtkneoß, as described by Nicolas et al., Cold Spring Harb., Conf. Cell Prolif. 10: 469-485 (1983) confers geneticin resistance, so it can be used as a marker for cotransfection, as it was in this experiment.

Following a procedure similar but not identical to that of Corsao et al., Somatic Cell Molec. Genet 7: 603-616 (1981), total genomic DNA and the plasmid wer cotransfected. The genomic DNA (60 µg) and plasmid DNA (6 µg) were mixed in 940 µl of 1 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, after which 310 µl of 1M $CaCl_2$ was added. This solution was slowly added, under constant agitation, to 1.25 ml of 2xHBS (50 mM HEPES, 280 mM NaCl 1.5 mM Na_2HPO_4, adjusted to pH 7.1 with NaOH). The calcium phosphate DNA precipitates were allowed to form for 30-45 minutes at room

temp rature, after which they were applied to 80 cm^2 tissue culture flasks which had been seeded 24 hours previously with $3x10^6$ MEL2.2 cells, in 22.5 ml of melanoma culture medium (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum. After 24 hours, the medium was replaced. Forty eight hours after transfection, the cells were harvested and seeded at $4x10^6$ cells per 80 cm^2 flask in melanoma culture medium supplemented with 2 mg/ml of geneticin. The geneticin serves as a selection marker.

<u>Example 16</u>

Thirteen days after transfection, geneticin-resistant colonies were counted, harvested, and cultured in nonselective medium for 2 or 3 days. Transfected cells were then plated in 96-well microplates at 200 cells/well in 200 ul of culture medium with 20% fetal calf serum (FCS) in order to obtain approximately 30 growing colonies per well. The number of microcultures was aimed at achieving redundancy, i.e., such that every independent transfectant should be represented at least four times.

After 10 days, wells contained approximately $6x10^4$ cells. These cells were detached, and 1/3 of each microculture was transferred to a duplicate plate. After 6 hours, i.e., after readherence, medium was removed and 1500 anti-E CTL (CTL 82/30), were added to each well in 100 μl of CTL culture medium with 35 U/ml of IL-2. One day later, the supernatant (50 μl) was harvested and examined

for TNF concentration, for reasons set forth in the following example.

Example 17

The size of the mammalian genome is $6x10^6$ kb. As the average amount of DNA integrated in each drug-resistant transfectant was expected to be about 200 kb, a minimum of 30,000 transfectants would need to be examined to ascertain whether antigen E had been transfected. Prior work with murine cells had shown that when a CTL stimulation assay was used, groups containing only 3% of cells expressing the antigen of interested could be identified. This should reduce the number of assays by a factor of 30. While an anti-E CTL assay, as described supra, in mixed E^+/E^- cells was helpful, it was not sufficient in that consistent results could not be obtained.

As a result, an alternative test was devised. Stimulation of CTLs was studied by release of tumor necrosis factor ("TNF") using well known methodologies which need not be repeated here. As described in Example 15, 1500 CTL 82/30 cells had been added per well of transfectants. These CTLs were collected 6 days after stimulation. As indicated supra, after 1/3 of the cells in each well had been removed and the remaining 2/3 ($4x10^4$) had readhered, the CTLs and IL-2 were added thereto. The 50 μl of supernatant was removed 24 hours later and transferred to a microplate containing $3x10^4$ W13 (WEHI-164 clone 13;

Espevik et al., J. Immunol. Meth. 95: 99-105 (1986)) cells in 50 μl of W13 cultur medium (RPMI-1640, supplemented with L-arginine (116 mg/l), L-asparagine (36 mg/l), L-glutamine (216 mg/l), and 10% FCS supplemented with 2 μg of actinomycin D at 37% in an 8% CO_2 atmosphere. The cell line W13 is a mouse fibrosarcoma cell line sensitive to TNF. Dilutions of recombinant TNF-β in RPMI 1640 were added to target cell controls.

The W13 cultures were evaluated after 20 hours of incubation, and dead cell percentage was measured using an adaptation of the colorimetric assay of Hansen et al., J. Immunol. Meth. 119: 203-210 (1989). This involved adding 50 ml of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide at 2.5 mg/ml in PBS, followed by two hours of incubation at 37°C. Dark blue formazan crystals were dissolved by adding 100 μl of lysis solution (1 volume N,N dimethyl formamide mixed at 37°C with two volumes of water containing 30% (w/v) sodium dodecyl sulphate, at pH 4.7 from 1.6% acetic acid and 2.5% 1N HCl). Plates were incubated at 37°C overnight, and ODs were taken at 570 nm using 650 nm as control. Dead cell percentage was determined via the formula:

$$100 \times \left[1 - \frac{100-(OD_{570} \text{ sample well})}{OD_{570} \text{ well + medium}} \right]$$

following Espevik et al., J. Immunol. Meth. 95: 99-105 (1986). The results showed that even when the ratio of E^+/E^- cells was as low as 1/45, significant production of TNF was observed, thus showing active CTLs. This led to the decision to test the drug resistant transfectants in groups of 30.

Example 18

Cells were tested for TNF production as discussed in Example 17, supra. A total of 100 groups of E^- cells ($4x10^6$ cells/group) were tested following transfection, and $7x10^4$ independent geneticin resistant transfectants were obtained, for an average of 700 per group. Only one group of transfected cells led to a microculture which caused anti-E antigen CTL clone 82/30 to produce TNF. Of 300 clones tested, 8 were positive. These clones were then tested for lysis by anti-E CTL, using the standard ^{51}Cr release assay, and were found to be lysed as efficiently as the original E^+ cell line. The transfectant E.T1, discussed herein, had the same lysis pattern as did MEL2.2 for CTLs against antigens B,C,D and F.

The fact that only one transfectant presented the antigen out of 70,000 geneticin resistance transfectants may at first seem very low, but it is not. The work described supra for P815 showed an average frequency of 1/13,000. Human DNA recipient MEL2.2 appears to integrate 5 times less DNA than P1.HTR.

Example 19

Once transfectant E.T1 was found, analysis had to address several questions including whether an E^+ contaminant of the cell population was the cause. The analysis of antigen presentation, described supra, shows that E.T1 is B^- and C^-, just like the recipient cell MEL2.2. It was also found to be $HPRT^-$, using standard selection procedures. All E^+ cells used in the work described herein, however, were $HPRT^+$.

It was also possible that an E^+ revertant of MEL2.2 was the source for E.T1. To test this, the observation by Perucho et al., Cell 22: 309-317 (1980), that cotransfected sequences usually integrate together at a single location of recipient genome was employed. If antigen E in a transfectant results from cotransfec-tion with pSVtkneoß, then sequences should be linked and deletion of the antigen might also delete the neighboring pSVtkneoß sequences. Wölfel et al., supra, has shown this to be true. If a normally E^- cell is transfected with pSVtkneoß, then sequences should be linked and deletion of the antigen might also delete the neighboring pSVtkneoß sequences. If a normally E^+ cell transfected with pSVtkneoß is E.T1, however, "co-deletion" should not take place. To test this, the transfectant E.T1 was subjected to immunoselection with 82/30, as described supra. Two antigen loss variants were obtained, which resisted lysis by this CTL. Neither of these had lost geneticin

resistance; however, Southern blot analysis showed loss of several neo^r sequences in the variants, showing close linkage between the E gene and neo^r gene in E.T1, leading to the conclusion that E.T1 was a transfectant.

Example 20

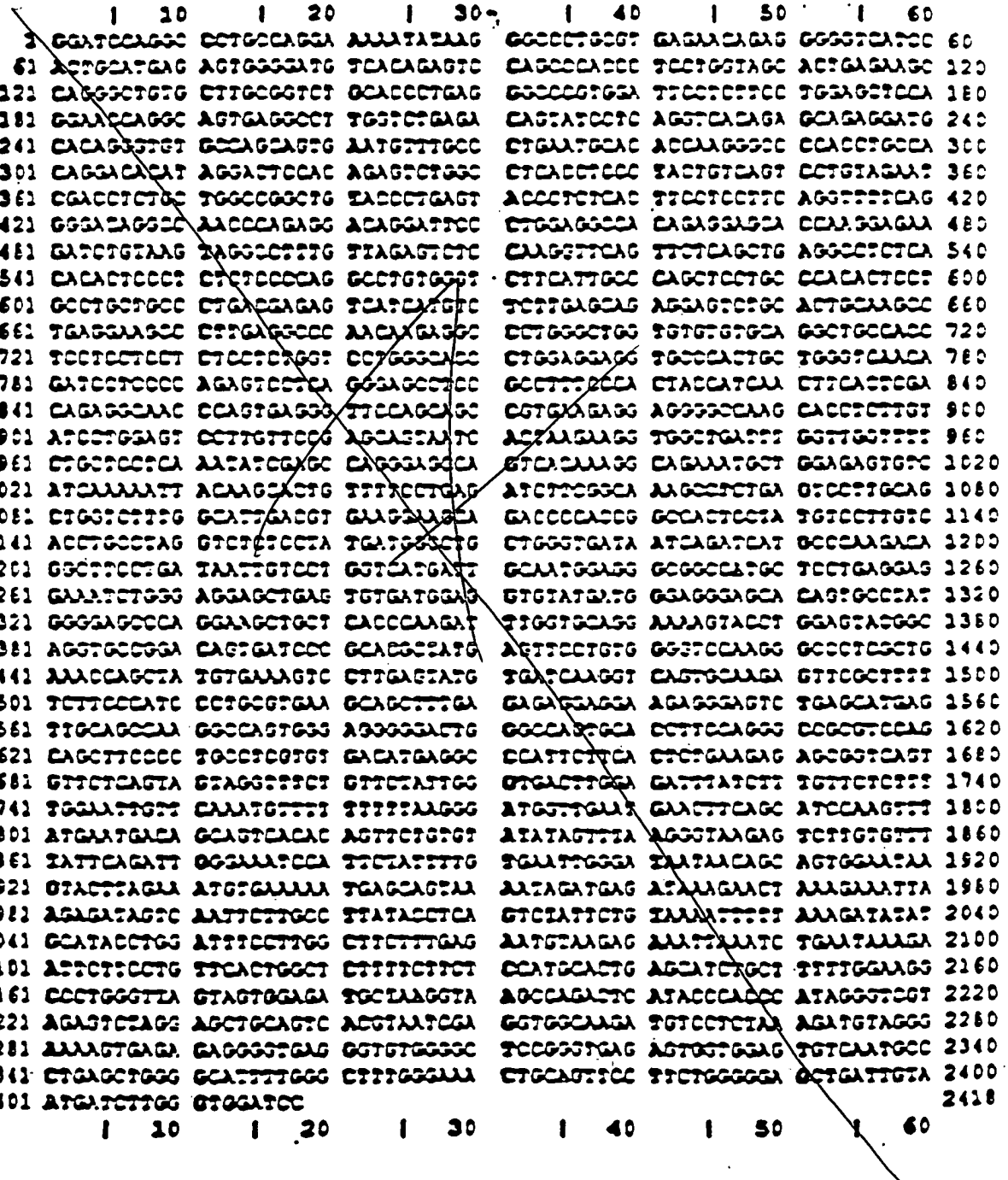
The E^+ subclone MZ2-MEL 4B was used as a source of DNA for preparation of a cosmid library. This library of nearly 700,000 cosmids was transfected into MZ2-MEL 2.2 cells, following the cosmid transfection protocols described supra.

By packaging the DNA of cosmid transfectants directly into lambda phase components, it is sometimes possible to retrieve cosmids that contain the sequences of interest. This procedure was unsuccessful here, so we rescued the transfected sequence by ligating DNA of the transfectant to appropriate restriction fragments of cosmid vector pTL6. This was tried with two transfectants and was successful with one of them. One cosmid, referred to as B3, was recovered from this experiment, and subjected to restriction endonuclease digestion via XmaI, or by BamHI digestion of a large, 12 kb XmaI transfected fragment. The fragments were cloned into vector pTZ 18R, and then transfected into MEL2.2. Again, TNF production was the measure by which successful transfection was determined. The experiments led to the determination of a gene sequence capable of transfecting antigen E on the 12 kb XmaI

fragment, and then on the 2.4 kb fragment of BamHI digestion of the 12 kb segment.

The 2.4 kb fragment hybridizes with a 2.4 kb fragment from MZ2-MEL and with a T cell clone of patient MZ-2, as determined by Southern Blots (BamHI/SmaI digested DNA). The band is absent from E^- antigen loss variants of MZ2-MEL, as seen in Figure 12.

The sequence for the E antigen precursor gene has been determined, and is presented herein: (SEQ ID NO: 7)



Exampl 21

After the 2.4 kb genomic segment had been identified, studies were carried out to determine if an "E^+" subline expressed any homologous DNA. Cell line MZ2-MEL 3.0 was used as a source, and a cDNA library was prepared from its mRNA, using art known techniques. The 2.4 kb segment was used as a probe, and mRNA of about 1.8 kb was identified as homologous, using Northern blot analysis. When cDNA was screened, clones were obtained showing almost complete identity to parts of the 2.4 kb fragment. Two exons were thus identified. An additional exon was located upstream of these, via sequencing segments of cosmid B3 located in front of the 2.4 kb BamHI fragment. The gene extends over about 4.5 kb, as shown in Figure 8. The starting point of the transcribed region was confirmed using PCR for the 5′ end of the cDNA. The three exons comprise 65, 73, and 1551 base pairs. An ATG is located at position 66 of exon 3, followed by an 828 base pair reading frame.

Example 22

To determine if smaller segments of the 2.4 kb fragment could transfer the expression of antigen E, smaller pieces corresponding to the larger gene were prepared, using art recognized techniques, and transferred into E^- cells. Figure 8 shows the boundaries of the three segments.

Transfer of antigen expression in this manner indicates that the gene codes for the antigen precursor, rather than coding for a protein which activates the antigen.

<u>Example 23</u>

The probing of cDNA described <u>supra</u> revealed, surprisingly, two different but closely related cDNAs. These cDNAs, when tested, did not transfer expression of antigen E, but they do show substantial homology to the first cDNA segment. The three segments, appear to indicate a newly recognized family of genes, referred to as "MAGE" for "melanoma antigen". In Figure 9, "mage -1" directs expression of the antigen from MZ2 cells. Portions of the third exon of each gene are presented in Figure 9. The second and third sequences are more closely related to each other than the first (18.1 and 18.9% difference compared to the first; 12% with each other). Out of 9 cDNA clones obtained, three of each type were obtained, suggesting equal expression. "MAGE" as used hereafter refers to a family of molecules, and the nucleic acids coding for them. These nucleic acids share a certain degree of homology and are expressed in tumor cells including several types of human tumor cells as well as in human tumors. The family is referred to as "MAGE" because the first members were identified in human melanoma cells. As the experiments which follow indicate, however, the members of the MAGE family are not at all restricted to melanoma tumors;

rather, MAGE r fers to a family of tumor rejection antigen precursors and the nucleic acid sequences coding therefore. The antigens resulting therefrom are referred to herein as "MAGE TRAs" or "melanoma antigen tumor rejection antigens"

Example 24

Experiments with mouse tumors have demonstrated that new antigens recognized by T cells can result from point mutations that modify active genes in a region that codes for the new antigenic peptide. New antigens can also arise from the activation of genes that are not expressed in most normal cells. To clarify this issue for antigen MZ2-E, the mage-1 gene present in the melanoma cells was compared to that present in normal cells of patient MZ2. Amplification by polymerase chain reaction (PCR) of DNA of phytohemagglutinin-activated blood lymphocytes using primers surrounding a 1300 bp stretch covering the first half of the 2.4 kb fragment was carried out. As expected, a PCR product was obtained whereas none was obtained with the DNA of the E^- variant. The sequence of this PCR product proved identical to the corresponding sequence of the gene carried by the E^+ melanoma cells. Moreover, it was found that antigen MZ2-E was expressed by cells transfected with the cloned PCR product. This result suggests that the activation of a gene normally silent is responsible for the appearance of tumor rejection antigen MZ2-E.

Exampl 25

In order to evaluate the expression of gene mage-1 by various normal and tumor cells, Northern blots were hybridized with a probe covering most of the third exon. In contrast with the result observed with human tumor cell line MZ2-MEL 3.0, no band was observed with RNA isolated from a CTL clone of patient MZ2 and phytohemagglutinin-activated blood lymphocytes of the same patient. Also negative were several normal tissues of other individuals (Figure 10 and Figure 11). Fourteen melanoma cell lines of other patients were tested. Eleven were positive with bands of varying intensities. In addition to these culture cell lines, four samples of melanoma tumor tissue were analyzed. Two samples, including a metastasis of patient MZ2 proved positive, excluding the possibility that expression of the gene represented a tissue culture artefact. A few tumors of other histological types, including lung tumors were tested. Most of these tumors were positive (Figures 10 and 11). These results indicated that the MAGE gene family is expressed by many melanomas and also by other tumors. However, they provided no clear indication as to which of genes mage-1, 2 or 3 were expressed by these cells, because the DNA probes corresponding to the three genes cross-hybridized to a considerable extent. To render this analysis more specific, PCR amplification and hybridization with highly specific oligo- nucleotide probes were used. cDNAs were obtained and amplified by PCR using oligonucleotide primers

corresponding to sequences of exon 3 that wer identical for the three MAGE genes discussed herein. The PCR products were then tested for their ability to hybridize to three other oligonucleotides that showed complete specificity for one of the three genes (Figure 9). Control experiments carried out by diluting RNA of melanoma MZ2-MEL 3.0 in RNA from negative cells indicated that under the conditions used herein the intensity of the signal decreased proportionally to the dilution and that positive signals could still be detected at a dilution of 1/300. The normal cells (lymphocytes) that were tested by PCR were confirmed to be negative for the expression of the three MAGE genes, suggesting therefore a level of expression of less than 1/300th that of the MZ2 melanoma cell line (Figure 11). For the panel of melanoma cell lines, the results clearly showed that some melanomas expressed MAGE genes mage 1, 2 and 3 whereas other expressed only mage-2 and 3 (Figures 11 and 10). Some of the other tumors also expressed all three genes whereas others expressed only mage-2 and 3 or only mage-3. It is impossible to exclude formally that some positive PCR results do not reflect the expression of one of the three characterized MAGE genes but that of yet another closely related gene that would share the sequence of the priming and hybridizing oligo-nucleotides. It can be concluded that the MAGE gene family is expressed by a large array of different tumors and that these genes are silent in the normal cells tested to this point.

Exammple 26

The availability of a sequence that transfects at high efficiency and efficiently expresses a TRAP made it possible to search for the associated major histocompatibility complex (MHC) class I molecule. The class I specificities of patient MZ2 are HLA-A1, A29, B37, B44 and C6. Four other melanomas of patients that had A1 in common with MZ2 were cotransfected with the 2.4 kb fragment and pSVtkneoß. Three of them yielded neo^r transfectants that stimulated TNF release by anti-E CTL clone 82/30, which is CD8+ (Figure 10). No E- transfectant was obtained with four other melanomas, some of which shared A29, B44 or C6 with MZ2. This suggests that the presenting molecule for antigen MZ2-E is HLA-A1. In confirmation, it was found that, out of 6 melanoma cell lines derived from tumors of HLA-A1 patients, two stimulated TNF release by anti-E CTL clone 82/30 of patient MZ2. One of these tumor cell lines, MI13443-MEL also showed high sensitivity to lysis by these anti-E CTL. These two melanomas were those that expressed mage-1 gene (Figure 13). Eight melanomas of patients with HLA haplotypes that did not include A1 were examined for their sensitivity to lysis and for their ability to stimulate TNF release by the CTL. None was found to be positive. The ability of some human anti-tumor CTL to lyse allogeneic tumors sharing an appropriate HLA specificity with the original tumor has been reported previously (Darrow, et al., J. Immunol. 142: 3329 (1989)). It is quite possible that antigenic peptides encoded by genes

mage 2 and 3 can also be presented to autologous CTL by HLA-A1 or other class I molecules, especially in view of the similar results found with murine tumors, as elaborated upon supra.

Example 27

As indicated supra, melanoma MZ2 expressed antigens F, D and A', in addition to antigen E. Following the isolation of the nucleic acid sequence coding for antigen E, similar experiments were carried out to isolate the nucleic acid sequence coding for antigen F.

To do this, cultures of cell line MZ2-MEL2.2, an E^- cell line described supra, were treated with anti-F CTL clone 76/6, in the same manner described for treatment with anti-E CTL clones. This resulted in the isolation of an F antigen loss variant, which was then subjected to several rounds of selection. The resulting cell line, "MZ2-MEL2.2.5" was completely resistant to lysis by anti-F CTLs, yet proved to be lysed by anti-D CTLs.

Again, following the protocols set forth for isolation of antigen -E precursor DNA, the F^- variant was transfected with genomic DNA from F^+ cell line MZ2-MEL3.0. The experiments yielded 90,000 drug resistant transfectants. These were tested for MZ2-F^- expression by using pools of 30 cells in the TNF detection assay elaborated upon supra. One pool stimulated TNF release by anti-F CTLs, and was cloned. Five of 145 clones were found to stimulate anti-

F CTLs. Lysis assays, also following protocols described supra, confirmed (i) expression of the gene coding for antigen F, and (ii) presentation of antigen F itself.

Example 28

Following identification of F^+ cell lines, the DNA therefrom was used to transfect cells. To do this, a cosmid library of F^+ cell line MZ2-MEL.43 was prepared, again using the protocols described supra. The library was divided into 14 groups of about 50,000 cosmids, and DNA from each group was transfected into MZ2-MEL2.2.5. Transfectants were then tested for their ability to stimulate TNF release from anti-F CTL clone 76/6. Of 14 groups of cosmids, one produced two independent transfectants expressing antigen F; a yield of two positives out of 17,500 geniticin resistant transfectants.

Example 29

The existence of a gene family was suggested by the pattern observed on the Southern blot (Figure 12). To do this, the 2.4 kb BamHI fragment, which transferred the expression of antigen M22-E, was labelled with 32p and used as a probe on a Southern Blot of BamHI digested DNA of E + cloned subclone M22-MEL2.2. Hybridization conditions included 50 $\mu l/cm^2$ of 3.5xSSC, 1xDenhardt's solution; 25 mM sodium phosphate buffer (pH 7.0), 0.5% SDS, 2mM EDTA, where the 2.4 kb probes had been labelled with [α^{32}p]dCTP (2-3000

Ci/mole), at $3x10^6$ cpm/ml. Hybridization was carried out for 18 hours at 65°C. After this, the membranes were washed at 65°C four times for one hour each in 2xSSC, 0.1% SDS, and finally for 30 minutes in 0.1xSSC, 0.1% SDS. To identify hybridization, membranes were autoradiographed using Kodak X-AR film and Kodak X-Omatic fine intensifying screens.

In the following examples, whenever "hybridization" is referred to, the stringency conditions used were similar to those described supra. "Stringent conditions" as used herein thus refers to the foregoing conditions; subject to routine, art recognized modification.

Example 30

The cDNA coding for mage 4 was identified from a sample of the human sarcoma cell line LB23-SAR. This cell line was found to not express mage 1, 2 or 3, but the mRNA of the cell line did hybridize to the 2.4 kb sequence for mage 1. To study this further, a cDNA library was prepared from total LB23-SAR mRNA, and was then hybridized to the 2.4 kb fragment. A cDNA sequence was identified as hybridizing to this probe, and is identified hereafter as mage 4.

Exampl 31

Experiments were carried out using PHA-activated lymphocytes from patient "MZ2", the source of the "MZ" cells discussed supra. An oligonucleotide probe which

showed homology to mage 1 but not mage 2 or 3 was hybridized with a cosmid library derived from the PHA activated cells. The size of the hybridizing BamHI cosmid fragment, however, was 4.5 kb, thus indicating that the material was not mage 1; however, on the basis of homology to mage 1-4, the fragment can be referred to as "mage 5". The sequence of MAGE 5 is presented in SEQ ID NO: 16.

Example 32

Melanoma cell line LB-33-MEL was tested. Total mRNA from the cell line was used to prepare cDNA, which was then amplified with oligos CHO9: (ACTCAGCTCCTCCCAGATTT), and CHO10: (GAAGAGGAGGGGCCAAG). These oligos correspond to regions of exon 3 that are common to previously described mage 1, 2 and 3.

To do this, 1 μg of RNA was diluted to a total volume of 20 μl, using 2 μl of 10x PCR buffer, 2 μl of each of 10 mM dNTP, 1.2 μl of 25 mM $MgCl_2$, 1 μl of an 80 mM solution of CHO9, described supra, 20 units of RNAsin, and 200 units of M-MLV reverse transcriptase. This was followed by incubation for 40 minutes at 42°C. PCR amplification followed, using 8 μl of 10x PCR buffer, 4.8 μl of 25 mM $MgCl_2$, 1 μl of CHO10, 2.5 units of Thermus acquaticus ("Taq") polymerase, and water to a total volume of 100 μl. Amplification was then carried out for 30 cycles (1 minute 94°C; 2 minutes at 52°C, 3 minutes at 72°C). Ten μl of each reaction were then size fractionated on agarose gel,

followed by nitrocellulose blotting. The product was found to hybridize with oligonucleotide probe CHO18 (TCTTGTATCCTGGAGTCC). This probe identified mage 1 but not mage 2 or 3. However, the product did not hybridize to probe SEQ 4 (TTGCCAAGATCTCAGGAA). This probe also binds mage 1 but not 2 and 3. This indicated that the PCR product contained a sequence that differed from mage 1, 2 and 3. Sequencing of this fragment also indicated differences with respect to mage 4 and 5. These results indicate a sequence differing from previously identified mage 1, 2, 3, 4 and 5, and is named mage 6.

Example 33

In additional experiments using cosmid libraries from PHA-activated lymphocytes of MZ2, the 2.4 kb mage 1 fragment was used as a probe and isolated a complementary fragment. This clone, however, did not bind to oligonucleotides specific for mage 1, 2, 3 or 4. The sequence obtained shows some homology to exon 3 of mage 1, and differs from mages 1-6. It is referred to as mage 7 hereafter. Additional screenings yielded mage 8-11.

Example 34

The usefulness of the TRAPs, as well as TRAs derived therefrom, was exemplified by the following.

Exon 3 of mage 1 was shown to transfer expression of antigen E. As a result, it was decided to test whether

synthetic peptides derived from this exon 3 could be used to confer sensitivity to anti-E CTL.

To do this, and using standard protocols, cells normally insensitive to anti-E/CTLs were incubated with the synthetic peptides derived from Exon 3.1. Using the CTL lytic assays described supra on P815A, and a peptide concentration of 3 mM, the peptide Glu-Ala-Asp-Pro-Thr-Gly-His-Ser-Tyr (SEQ ID NO:26) was shown to be best. The assay showed lysis of 30%, indicating conferring of sensitivity to the anti-E CTL.

Example 35

Nucleic acid sequences referred to as "smage" were isolated from murine cells. Using the protocols described supra, a cosmid library was prepared from the DNA of normal DBA/2 kidney cells, using cosmid vector C2RB. As a probe, the 2.4 kb BamHI fragment of MAGE-1 was used. The DNA was blotted to nylon filters, and these were washed in 2xSSC at 65°C to identify the smage material.

Example 36

Further tissue samples were tested for the presence of MAGE genes, using the protocols discussed supra. Some of these results follow.

There was no expression of the MAGE genes in brain or kidney tumor tissue. Colon tumor tissue showed expression of MAGE 1, 2, 3 and 4, although not all tumors tested showed expression of all MAGE genes. This is also true for

pancreatic tumor (MAGE 1); non-small cell lung (MAGE 1, 2, 3 and 4), prostate (MAGE 1), sarcomas (MAGE 1, 2, 3 and 4), breast (MAGE 1, 2 and 3), and larynx (MAGE 1 and 4).

The foregoing disclosure, including the examples, places many tools of extreme value in the hands of the skilled artisan. To begin, the examples identify and provide a methodology for isolating nucleic acid molecules which code for tumor rejection antigen precursors as well as the nucleic acid molecules complementary thereto. It is known that DNA exists in double stranded form, and that each of the two strands is complementary to the other. Nucleic acid hybridization technology has developed to the point where, given a strand of DNA, the skilled artisan can isolate its complement, or synthesize it.

"Nucleic acid molecule" as used herein refers to all species of DNA and RNA which possess the properties discussed supra. Genomic and complementary DNA, or "cDNA" both code for particular proteins, and as the examples directed to isolation of MAGE coding sequences show, this disclosure teaches the artisan how to secure both of these.

Similarly, RNA molecules, such as mRNA can be secured. Again, with reference to the skilled artisan, once one has a coding sequence in hand, mRNA can be isolated or synthesized.

Complementary sequences which do not code for TRAP, such as "antisense DNA" or mRNA are useful, e.g., in

probing for the coding sequence as well as in methodologies for blocking its expression.

It will also be clear that the examples show the manufacture of biologically pure cultures of cell lines which have been transfected with nucleic acid sequences which code for or express the TRAP molecules. Such cultures can be used as a source for tumor rejection antigens, e.g., or as therapeutics. This aspect of the invention is discussed infra.

Cells transfected with the TRAP coding sequences may also be transfected with other coding sequences. Examples of other coding sequences include cytokine genes, such as interleukins (e.g., IL-2 or IL-4), or major histo-compatibility complex (MHC) or human leukocyte antigen (HLA) molecules. Cytokine gene transfection is of value because expression of these is expected to enhance the therapeutic efficacy of the biologically pure culture of the cells in vivo. The art is well aware of therapies where interleukin transfectants have been administered to subjects for treating cancerous conditions. In a particularly preferred embodiment, cells are transfected with sequences coding for each of (i) a TRAP molecule, (ii) an HLA/MHC molecule, and (iii) a cytokine.

Transfection with an MHC/HLA coding sequence is desirable because certain of the TRAs may be preferentially or specifically presented only by particular MHC/HLA molecules. Thus, where a recipient cell already expresses the MHC/HLA molecule associated with presentation of a TRA,

additional transfection may not be necessary although further transformation could be used to cause over-expression of the antigen. On the other hand, it may be desirable to transfect with a second sequence when the recipient cell does not normally express the relevant MHC/HLA molecule. It is to be understood, of course, that transfection with one additional sequence does not preclude further transfection with other sequences.

The term "biologically pure" as used in connection with the cell line described herein simply means that these are essentially free of other cells. Strictly speaking, a "cell line" by definition is "biologically pure", but the recitation will establish this fully.

Transfection of cells requires that an appropriate vector be used. Thus, the invention encompasses expression vectors where a coding sequence for the TRAP of interest is operably linked to a promoter. The promoter may be a strong promoter, such as those well known to the art, or a differential promoter, i.e., one which is operative only in specific cell types. The expression vectors may also contain all or a part of a viral or bacterial genome, such as vaccinia virus or BCG. Such vectors are especially useful in preparing vaccines.

The expression vectors may incorporate several coding sequences, as long as the TRAP sequence is contained therein. The cytokine and/or MHC/HLA genes discussed <u>supra</u> may be included in a single vector with the TRAP sequence. Where this is not desired, then an expression system may be

provid d, where two or more separate vectors are used where each coding sequence is operably linked to a promoter. Again, the promoter may be a strong or differential promoter. Co-transfection is a well known technique, and the artisan in this field is expected to have this technology available for utilization. The vectors may be constructed so that they code for the TRA molecule directly, rather than the TRAP molecule. This eliminates the need for post-translational processing.

As the foregoing discussion makes clear, the sequences code for "tumor rejection antigen precursors" ("TRAPs") which, in turn, are processed into tumor rejection antigens ("TRAs"). Isolated forms of both of these categories are described herein, including specific examples of each. Perhaps their most noteworthy aspect is as vaccines for treating various cancerous conditions. The evidence points to presentation of TRAs on tumor cells, followed by the development of an immune response and deletion of the cells. The examples show that when various TRAs are administered to cells, a CTL response is mounted and presenting cells are deleted. This is behavior characteristic of vaccines, and hence TRAPs, which are processed into TRAs, and the TRAs themselves may be used, either alone or in pharmaceutically appropriate compositions, as vaccines. Similarly, presenting cells may be used in the same manner, either alone or as combined with ingredients to yield pharmaceutical compositions. Additional materials which may be used as vaccines include

isolated cells which present the TRA molecule on their surface, as well as TRAP fragments, mutated viruses, especially etiolated forms, and transfected bacteria. "Fragments" as used herein refers to peptides which are smaller than the TRA, but which possess the properties required of a vaccine, as discussed supra. Another vaccine comprises or consists of complexes of TRA and HLA molecule. Vaccines of the type described herein may be used preventively, i.e., via administration to a subject in an amount sufficient to prevent onset of a cancerous condition.

The generation of an immune response, be it T-cell or B-cell related, is characteristic of the effect of the presented tumor rejection antigen. With respect to the B-cell response, this involves, inter alia, the generation of antibodies to the TRA, i.e., which specifically bind thereto. In addition, the TRAP molecules are of sufficient size to render them immunogenic, and antibodies which specifically bind thereto are a part of this invention. These antibodies may be polyclonal or monoclonal, the latter being prepared by any of the well recognized methodologies for their preparation which need not be repeated here. For example, mAbs may be prepared using an animal model, e.g., a Balb/C mouse or in a test tube, using, .g., EBV transformants. In addition, antiserum may be isolated from a subject afflicted with a cancerous condition where certain cells present a TRA. Such

antibodies may also be generated to epitopes defined by the interaction of TRA and HLA/MHC molecules.

Review of the foregoing disclosure will show that there are a number of facets to the system which may be referred to as "tumor rejection antigen presentation and recognition". Recognition of these phenomena has diagnostic consequences. For example, the existence of specific CTL clones, or antibodies to the TRA makes it possible to diagnose or monitor cancerous conditions (explained infra), by monitoring the CTLs in a sample from a subject, binding of antibodies to TRAs, or the activity of anti-TRA CTLs in connection with subject samples. Similarly, the expression of nucleic acid molecules for TRAPs can be monitored via amplification (e.g., "polymerase chain reaction"), anti-sense hybridization, probe technologies, and so forth. Various subject samples, including body fluids (blood, serum, and other exudates, e.g.), tissues and tumors may be so assayed.

A particular manner of diagnosis is to use an adaptation of the standard "tuberculin test" currently used for diagnosis of tuberculosis. This standard skin test administers a stable form of "purified protein derivative" or "PPD" as a diagnostic aid. In a parallel fashion, TRAs in accordance with this invention may be used in such a skin test as a diagnostic aid or monitoring method.

The term "cancerous condition" is used herein to embrace all physiological events that commence with the initiation of the cancer and result in final clinical

manifestation. Tumors do not spring up "ab initio" as visible tumors; rather ther are various events associated with the transformation of a normal cell to malignancy, followed by development of a growth of biomass, such as a tumor, metastasis, etc. In addition, remission may be conceived of as part of "a cancerous condition" as tumors seldom spontaneously disappear. The diagnostic aspects of this invention include all events involved in carcinogenesis, from the first transformation to malignancy of a single cell, through tumor development and metastasis, as well as remission. All are embraced herein.

Where "subject" is used, the term embraces any species which can be afflicted with a cancerous condition. This includes humans and non-humans, such as domesticated animals, breeding stock, and so forth.

There are therapeutic aspects of this invention as well. The efficacy of administration of effective amounts of TRAPs and TRAs as vaccines has already been discussed _supra_. Similarly, one may develop the specific CTLs _in vitro_ and then administer these to the subject. Antibodies may be administered, either polyclonal or monoclonal, which specifically bind to cells presenting the TRA of interest. These antibodies may be coupled to specific antitumor agents, including, but not being limited to, methotrexate radio-iodinated compounds, toxins such as ricin, other cytostatic or cytolytic drugs, and so forth. Thus, "targeted" antibody therapy is included herein, as is the

application of deletion of the cancerous cells by the use of CTLs.

The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, it being recognized that various modifications are possible within the scope of the invention.